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Expression of Immunoglobulin Genes Tandem in Eukaryotic Cells Under the Control of T7 Bacteriophage RNA Polymerase

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ABSTRACT

A tandem of recombinant mouse/human immunoglobulin (Ig) genes was constructed and inserted into the plasmid pGEM1 under the control of T7 phage RNA polymerase promoter. Sp2/0 lymphoid cell line and Chinese Hamster Ovary (CHO) cells were used as the targets for gene transfection. Both cell lines contained in their genomes a T7 RNA polymerase gene modified with a nuclear-located signal derived from SV40 large T-antigen. Cell lines transfected with the gene tandem effectively synthesized mRNA (up to 9×10^3 bp) that hybridized with ϵ- and $\varkappa$-gene probes. Separate transcripts corresponding to mRNAs of individual heavy and light chains were not detected in either transfected cell line. It follows from these data that transcription in the transfected cells is controlled mainly by the T7 phage polymerase promoter. Both lymphoid and nonlymphoid cell lines transfected with the gene tandem synthesized the ϵ-heavy (70 kDa) and $\varkappa$-light (25 kDa) Ig polypeptide chains. Production of chimeric antibodies by the myeloma Sp2/0 cells was higher than that by the CHO cells. Individual clones synthesized up to 150 ng/mL chimeric IgE. However, only lymphoid Sp2/0 cells were capable of efficient secretion of the recombinant antibodies. The mechanism of translation of mRNA synthesized in eukaryotic cells by T7 phage RNA polymerase is discussed.

Index Entries: Recombinant antibodies; gene tandem expression; phage T7 RNA polymerase; CHO cell; Sp2/0 cell.

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INTRODUCTION

The expression of both heavy and light immunoglobulin chain genes is under the control of regulatory elements, promoters, and enhancers, which are housed, respectively, upstream and downstream of the transcription start site (for review, *see* ref. *1*). DNA binding transcription factors recognize these genetic elements and participate in transcription initiation (*2–4*).

The development of recombinant DNA techniques allows us to construct a heterologous transcription machinery exhibiting a high level of expression. Earlier, T7 phage RNA polymerase was used for expression of marker genes in eukaryotic cells (*5–8*). It has been shown (*9*) that T7 RNA polymerase and phage promoters have unique properties that prevent interference with the expression of host genes. The T7 promotor contains 23 bp that are not encountered in other prokaryotic and eukaryotic promotors (*9*). The T7 phage transcription system has been introduced into mammalian cells, and it has been demonstrated that unmodified T7 RNA polymerase is functionally active in the cytoplasm of mammalian cells infected with vaccinia vectors (*5*). However, the unmodified T7 RNA polymerase system has some disadvantages: it functions only transiently and is not able to penetrate from the cytoplasm to the nucleus. Later, it has been shown that nuclear targeting of the T7 phage RNA polymerase overcomes these failures (*8*). The lymphoid (Sp2/0) and nonlymphoid (CHO) cell lines transfected with the modified T7 RNA polymerase gene (*8*) provide for stable expression of this gene, with a predominant nuclear location of the expression product. In the present work, a complete tandem of immunoglobulin genes has been designed that consists of the variable (V) genes of light (L) and heavy (H) chains obtained from the mouse hybridoma PTF-02 (*10*), as well as the genes of human constant (C) regions. The entire tandem has been put under the T7 RNA polymerase promoter control. Eukaryotic cells that steadily produce the modified T7 RNA polymerase have been used as targets. Thus, conditions have been created to allow us to investigate the expression of the construct in both lymphoid and nonlymphoid cells.

MATERIALS AND METHODS

Immunoglobulin Genes

Clones containing the variable gene segments were from the hybridoma PTF-02 genome (obtained through the courtesy of F. Franek, Institute of Molecular Genetics, Prague). Both V genes were cloned and sequenced by us earlier (*11,12*). In addition to the coding regions, these genes contain both promoters and the sequences necessary for the gene splicing.

The clones containing constant region genes of light (κ-type) and heavy (ϵ-type) chains were obtained from human genomic DNA.

Cell Lines

The myeloma cell line Sp2/0 and nonlymphoid CHO cells were used for transfection. Both cell lines contained in their genome a semisynthetic gene of T7 RNA polymerase (*8*), and they steadily express this polymerase.

Cell Transfection

The recombinant immunoglobulin gene tandem was inserted into the plasmid pGEM1 under the control of T7 RNA polymerase promoter (*13*). This promoter contains a universal sequence of 23 bp not found in other prokaryotic or eukaryotic promoters (*9*). Simultaneously with Ca-phosphate transfection, cotransfection with the plasmid pSV2-neo was carried out, and clones were selected by growth in medium containing Geneticin G-418 (400 μg/mL, CHO cells; 1.5 mg/mL; Sp2/0 cells).

DNA Probes

*Bam*HI-*Pst*I fragment (2.1 kb) containing human C_ϵ gene and *Eco*RI-*Eco*RI fragment (2.6 kb) containing C_κ gene were labeled with ^{32}P, cloned in plasmid pUC19, and used as ^{32}P-labeled DNA probes.

Radioimmune Analysis

Rabbit antihuman whole IgE and anti-ϵ-chain antibodies were obtained from the Institute of Pulmonology (Berlin-Buch, Germany). These antibodies do not react with other types of immunoglobulin chains. Goat antibodies to rabbit IgG were obtained from the same source. ^{125}I-labeled protein A was from Amersham, England.

Enzymes and Reagents

Restriction endonucleases and other enzymes were obtained from the Institute of Applied Enzymology (Vilnius, Lithuania), agarose from Bio-Rad (USA), and other reagents from Sigma (USA).

RESULTS

Construction of Recombinant Plasmid pIG.6ek. Containing the Immunoglobulin Gene Tandem

In the first step, the V_H gene from mouse hybridoma and the human C_ϵ gene were inserted into the pGEM1 plasmid under the control of bacteriophage T7 RNA polymerase promoter (Fig. 1). The sticky ends of the

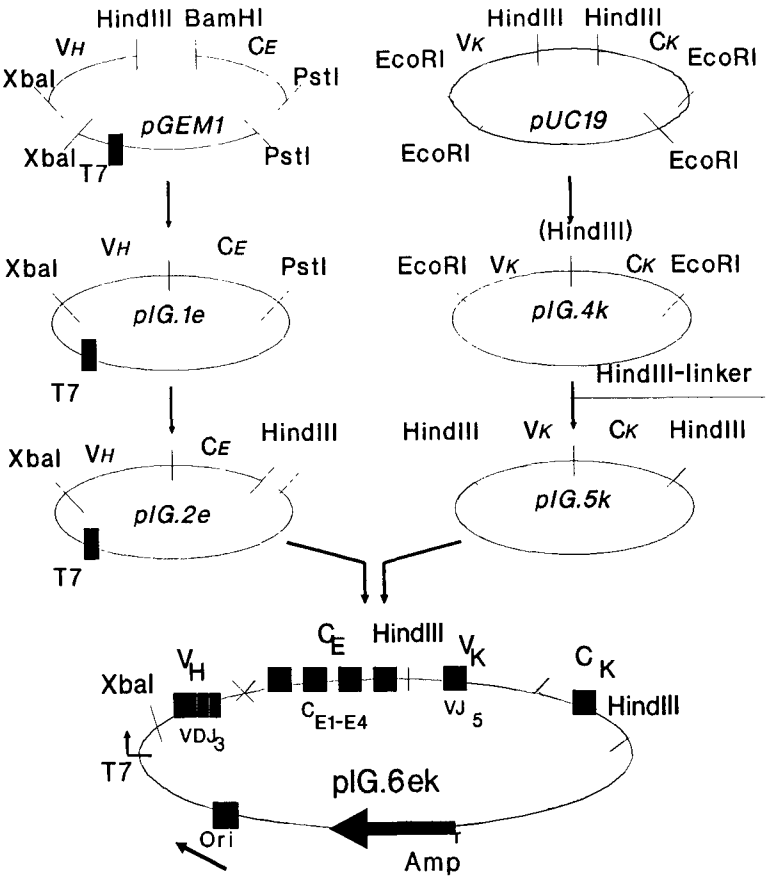

Fig. 1. Scheme of chimeric immunoglobulin gene construction. Variable heavy (V_H) and light kappa (V_x) chain genes obtained from mouse hybridoma were ligated with C epsilon (C_ϵ) and C kappa (C_x) genes, respectively. The gene tandem was placed under the control of T7 RNA polymerase promoter (*see text* for details).

*Hind*III fragment of the V_H gene and the *Bam*HI fragment of the C_ϵ gene were blunted with the Klenow fragment of DNA-polymerase and the intermediate ligated by its blunt ends. *E. coli* cells (strain DH5α) were transformed with a vector pIG.1e, containing both V_H and C_ϵ genes. The clones containing inserts were screened by the method described in (*14*) and analyzed using restriction endonucleases.

The mouse V_x gene (from the same hybridoma) and the human C_x gene were inserted into the pUC19 vector (Fig. 1). *E. coli* DH5α cells were transformed with the plasmid pIG.3k, and clones containing the ligation product were treated with *Hind*III. The sticky ends were blunted and then ligated. Plasmid pIG.4k (*see* Fig. 1) was cloned in *E. coli* JM103. *Eco*RI sites at both ends of V_xC_x genes tandem were replaced with *Hind*III sites. The final construct consisted of the V_xC_x gene fused to the 3′-end of the V_HC_ϵ

gene. The structure of the final pIG.6ek plasmid is shown in Fig. 1. Both sets of genes coding for heavy and light chains in the pIG.6ek plasmid contain native gene promoters, and do not contain internal or 3′-end enhancers. The sequence of the tandem of variable heavy and light immunoglobulin genes inserted into the expression vector is shown in Fig. 2.

Expression of Recombinant Antibodies in Lymphoid and Nonlymphoid Cells

The lymphoid (Sp2/0) and nonlymphoid (CHO) cells steadily produced the modified T7 RNA polymerase (*see* Materials and Methods). These cells were transfected with the plasmid pIG.6ek shown in Fig. 1. The Geneticin-resistant clones were selected and analyzed by hybridization to ^{32}P-labeled constant human ϵ- and $\varkappa$-genes (Fig. 3). This permitted the identification of transfectants that steadily synthesize mRNA coding for the heavy and light chains. The original cell lines do not synthesize these mRNA species.

By Northern blot analysis of mRNA synthesized by the transfected cells (not shown), we identified an extended mRNA species (8.5–9.0 $\times$ 10^3 bp). RNA bands corresponding to the mobilities of the individual mRNAs for light and heavy chains were not observed. Next, we examined the synthesis of light and heavy chains by these cell lines. The polypeptide chains were identified by electrophoresis of cellular extracts, Western blotting, and dot-blot analysis using anti-Ig rabbit antibodies and ^{125}I-labeled protein A. The transfected Sp2/0 and CHO clones synthesized both heavy (ϵ-type) and light ($\varkappa$-type) immunoglobulin chains (Fig. 4). We observed that some transfected cell lines synthesized the heavy and light chains in nearly equimolar amounts, whereas other lines produced the heavy chain preferentially (Fig. 4). Concerning the mass of expressed proteins, 70-kDa heavy and 25-kDa light chains were produced in both types of cell lines. A product corresponding to the heavy chain, but with larger mass was evident in some cell lines (in comparison with the natural ϵ-chain). The larger mass product may be the result of the presence of an alternative mRNA splicing pathway. The level of chimeric antibody production by the myeloma Sp2/0 cells was higher than that by the CHO cells. Individual clones synthesizing up to 150 ng/mL of chimeric antibodies were identified.

The activity of the antibodies was determined by measuring binding of the antigen (pig transferrin) (Fig. 5). The antigen was immobilized on nitrocellulose filters, and treated with antibodies present in cell extracts. Filter-bound antibodies were identified by exposure to radiolabeled protein A. Transfected cells of both types (Sp2/0 and CHO) synthesized functional antibodies capable of binding transferrin. We have also estimated the chimeric antibodies based on their reaction with rabbit antibodies to human IgE. The chimeric antibodies were present in the extracts

A

V_H

```
TAGATGTGTTTCCTGTGATTTCTAAAGTCTTATTGCTCTCTTATTGGAGACTCACACTAT    60
AGGAAGCCAGAGACCATGATGGTCTTACTTTAATAACCAAGGGCATTCATTATTTACCTT   120
CCCAAATTATGAAGGCTGGGCTGTCCTGCATGCAAATGCTTCTAACTCTAAGTTAAATCC   180
CCTCTTGGGGTGTGAAAGCTCACATCTCTCTCATTAGAGGTTGATCTTTGAGGAAAACAG   240
                  M  K  V  L  S  L  L  Y  L  L  T  A  I  P  G
GGTGTTGCCTAAAGGATGAAAGTGTTGAGTCTGTTGTACCTGTTGACAGCCATTCCTGGT   300

GAGTGTTGATATTTCATACATGTACCATGAGGGTTTTTCAAAACGTGTGATTGACCAAAA   360

                        I  L  S  T  V  Q  L  Q  E  S  G  P  G
TGGCCCTTCTTTTCTGAAGGTATCCTGTCTACTGTACAGCTTCAGGAGTCAGGACCTGGC   420

 L  V  K  P  S  Q  S  L  S  L  T  C  S  V  T  D  F  S  I  T
CTCGTGAAACCTTCTCAGTCTCTGTCTCTCACCTGCTCTGTCACTGACTTCTCCATCACC   480

 S  G  Y  Y  W  H  W  I  R  Q  F  P  G  N  K  L  E  W  M  G
AGTGGTTATTACTGGCACTGGATCCGGCAGTTTCCAGGAAACAAACTGGAATGGATGGGC   540

 Y  I  S  Y  D  G  S  N  G  Y  N  P  S  L  K  N  R  I  S  I
TACATAAGTTACGACGGTAGTAATGGATACAACCCCTCTCTCAAAAATCGAATCTCCATC   600

 T  R  D  T  S  K  N  Q  F  F  L  K  L  N  S  V  T  T  E  D
ACTCGTGACACATCTAAGAACCAGTTTTTCCTGAAGTTGAATTCTGTGACTACTGAGGAC   660

 T  A  T  I  I  C  T  R  G  D  G  T  H  F  F  T  Y  W  G  Q
ACAGCCACATATTACTGTACAAGAGGTGATGGTTACCACTTCTTTACTTACTGGGGCCAA   720

 G  T  L  V  T  V  S  A
GGGACTCTGGTCACTGTCTCTGCA
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Fig. 2. Sequences of V_H (**A**) and V_x (**B**) genes and 5′-upstream regions inserted into plasmid plG.6ek (*see* Fig. 1). The deduced amino acid sequences of leader (L) and variable (V) genes are shown. ATG triplets located in 5′-upstream regions of both V_H and V_x genes are underlined. The invariant sequences of intron segments of 3′-downstream regions are not shown.

of both types of transfected cells, but the secretion of the antibodies was greater by Sp2/0 cells (Fig. 6). Thus, the nonlymphoid CHO cells synthesized the functionally active chimeric IgE, but unlike the lymphoid cells, they do not effectively secrete these antibodies.

DISCUSSION

An entire gene tandem of a mouse/human chimeric Ig, including exons, appropriate introns, and a spacer between the genes of light and heavy chains, was placed under the control of the T7 RNA polymerase promoter. The transfected cells contained in their genome the modified semisynthetic gene for T7 RNA polymerase (*8*). The modification (*8*) of the polymerase consisted of replacement of the N-terminal segment with amino acids 124–133 of the SV40 viral large T-antigen, coded for by a synthetic nucleotide sequence. This sequence is the signal for nuclear locali-

B

V_x

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AGCTTTAGGAAACATAGAGGATTTGAACGGGGGAATGGGCCATATTTAGAGAGACAGATT     60
TCTAAATCTCTACTACCTAGAAAAAATGGTTTATGTAAAGAAACAGAAAGAAAGAACAAT    120
AATTGAGGCATAGCATGTATATTAGGCAGTATGTGTATAACATATATATTATATTCTCTA    180
TATGATAGAGTGATAATATGGCACAAATGGAAAAGAAAAAAATCAGGACTGTAGAGTCAG    240
TCCACTTAGCTGTTCAGAATACTTGCTATTCTTGCAAACGATCTGGGTTAGGTTCCCAGC    300
AAACACGTGAATATCTCCATCATTTGAACTTTAAAATCCTTTTCTGAATTCCCCTAGCAA    360
TAAATACATAGACAATGGGCATGCATACATACATCAAGAGGTAAGCACTCATGCACATAA    420
AATTACAGTTTTTGTTGTTGTTTTCATGTCATATTTTTTGAGAAGAGAATCAAACAGAAG    480
TTCCTCTTTGGTAGTCAAAAGAATCATAAATAGATTAAGATGAATTGGACAGTGCTGGGA    540
ATGAATGGAGTGGTGATGTACTATCTTGTCATTCCTTAGAAGATGTGCCTCCCAATATTT    600
TTCAATTTGAATTTTGATAAATACAGAAAGAGCTAGGTATAATCATAAACAAGAATGAAA    660
TTTTTTATGACCAGTGATATCTACAGATAACTATTAATGAACATTTTGAAGTTTCTGCGA    720
ACATGCAAATCCTGAAACTCCCTAGCATATACAACATATAGGAATAGAATGATGTACTGA    780
ACAAGGGGGAAATTTTTAAAAAGCATTAAATAAATGCTTTTTAAGACTATTTTTTGTGCC    840
TCCAAAGATGAGCACCTTCATTGGAGAAAAATCTGTCTGTGCTGGTCTGATGTAACAAAT    900
CAGAGGTTGTTACGCTTTGGTTGAGTTACTAAAATAAGCCTATTCTGCAGCTGTGCCCAG    960
CATTGTTCGATGAAGCTGATTTGCATGTGCTGAGATCATATTCTACTGCCCCAGAGATTT   1020

                                                  M  R  P  S
AATAATCTGATCATACACACTTCATTCTTCCTCAGGAGACGTTGTAGAAATGAGACCGTC   1080

   I  Q  F  L  G  S  C  C  S  G  F  M  V
TATTCAGTTCCTGGGCTCTTGTTGTTCTGGCTTCATGGTAAGGAGTTTAACATTGAATAT   1140

GCTAAAAAGAGTATGTGATCAGGAATTTCTGGTCCTTCAGAAAAATCTTCTTTGCATATA   1200

                                                  D  I  Q  M
ATTAATTTCATGGGGATTTGTGTTCTTTTTAATTATAGGTCCTCAGTGTGACATCCAGAT   1260

   T  Q  S  P  S  S  L  S  A  S  L  G  G  K  V  T  I  T  C  K
GACACAGTCTCCATCCTCACTGTCTGCATCTCTGGGAGGCAAAGTCACCATCACTTGCAA   1320

   A  V  Q  D  I  L  K  K  Y  I  P  W  F  Q  R  K  P  R  R  G
GGCAGTCCAAGACATTTTGAAGAAATATATACCTTGGTTCCAACGCAAGCCTAGAAGAGG   1380

   P  R  L  L  I  H  Y  T  S  T  L  Q  P  G  I  P  S  R  F  G
TCCTAGACTGCTCATACATTACACATCTACATTACAGCCAGGCATCCCATCAAGGTTCGG   1440

   G  S  G  S  G  K  L  F  L  R  H  Q  Q  L  E  D  E  Y  F  S
TGAAAGCGGGTCTGGAAAATTATTCCTTCGGCATCAGCAACTGGAGCCTGAATATTTTTC   1500

   T  F  Y  C  P  Q  Y  D  S  L  L  T  F  G  G  G  T  K  L  E
AACTTTTTATTGTCCACAGTATGATAGTCTTCTCACGTTCGGTGGTGGGACCAAGCTGGA   1560

   L  K  R
GCTGAAACGT
```

zation of the protein, allowing the modified polymerase to penetrate effectively from the cytoplasm into the nucleus and providing for nuclear transcription under T7 promoter control. The cells expressed the enzyme steadily. Using dot-blot hybridization, we observed the synthesis of mRNA for heavy and light chain in both types of transfected cells (lymphoid Sp2/0 and nonlymphoid CHO cells). The transcript size in the transfected CHO cells was close to 9×10^3 bp, which is slightly larger than the size of the gene tandem, and is indicative of effective transcription of the tandem under T7 promoter control. The phage T7 promoter can function

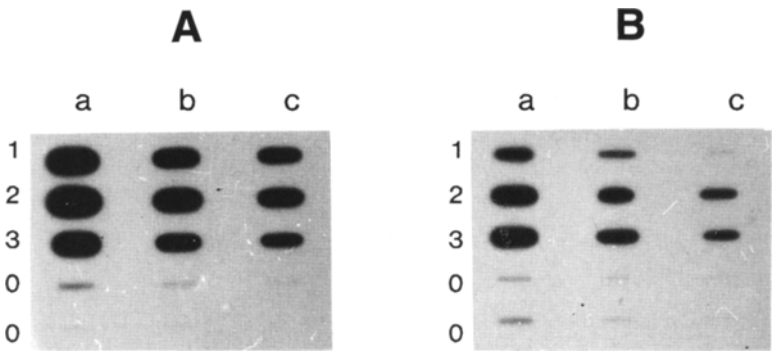

Fig. 3. Dot-blot hybridization analysis of mRNA synthesized by transfected Sp2/0 cell lines. (**A**) Hybridization with the ^{32}P-labeled human C_ϵ-gene probe. (**B**) Hybridization with the ^{32}P-labeled human $C_\varkappa$-gene probe. Arabic numerals refer to different cell lines. 200 ng (a), 100 ng (b), and 20 ng (c) of mRNA were used for analysis with appropriate probes. Untransfected Sp2/0 cells were used as a control (0). See ref. (*8*) for method.

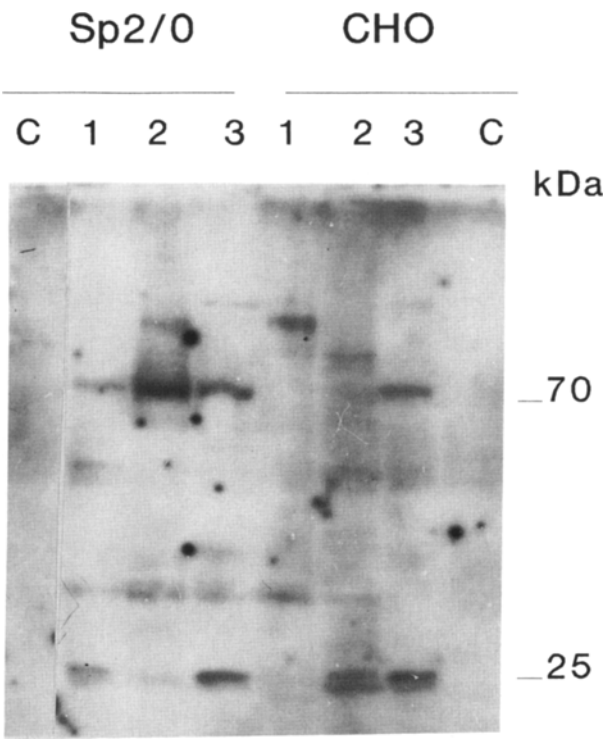

Fig. 4. Western blot analysis for heavy (ϵ) and light ($\varkappa$) chain synthesis by lymphoid (Sp2/0) and nonlymphoid (CHO) cell lines transfected with plasmid pIG.6ek Arabic numerals represent different cell lines. C, untransfected cell lines. Numbers to the right of the blots indicate approximate mol wt in kDa. Electrophoresis and Western blotting were as in (*27*).

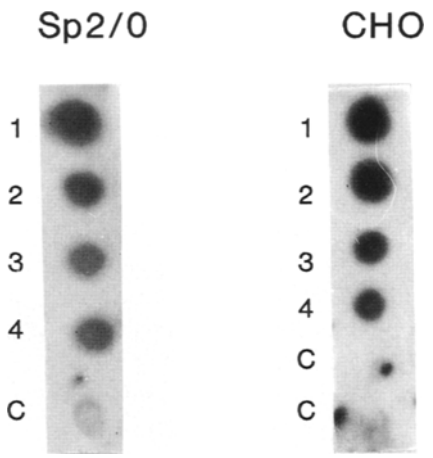

Fig. 5. Binding of the antigen (pig transferrin) with chimeric antibodies from lysates of transfected Sp2/0 and CHO cell lines (*1–4*). C, untransfected cells. Antigen (pig transferrin) was immobolized on nitrocellulose filters, and the filters were analyzed by dot-blotting using cell lysates as the source of the antibodies. Antigen–antibody complexes were detected with rabbit antihuman IgE antibodies and ^{125}I-labeled protein A.

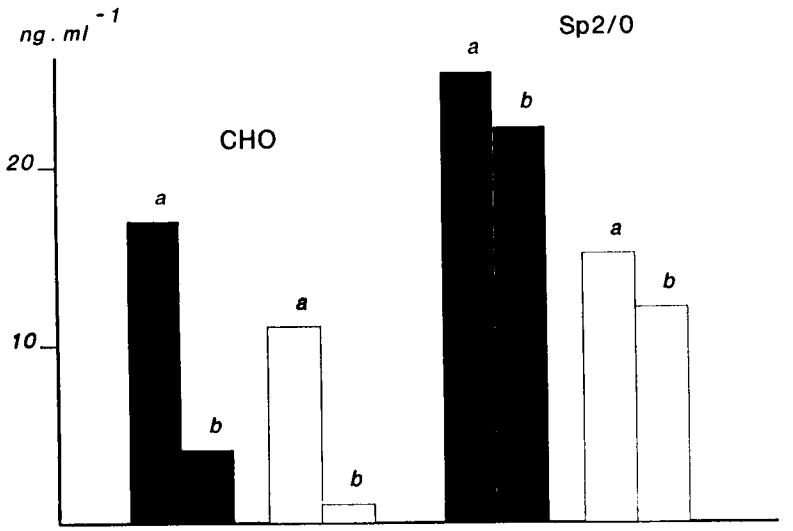

Fig. 6. Accumulation of chimeric antibodies in the cell extracts (***a***) and culture medium (***b***) of CHO and Sp2/0 cell lines transfected with plasmid pIG.6ek (5×10^6 cells/ml). Identification was by radioimmunoassay using anti-ε (filled bars) and antiwhole IgE antibody from hybridoma PTF-02 (open bars).

as a polymerase II promoter in mammalian cells (*6*). However, we did not find separate transcripts corresponding to the mRNAs of the light and heavy chains in either type of transfected cells, suggesting that transcription of both genes coding for the individual antibody subunits is controlled mainly by the T7 polymerase that recognizes its own promoter localized upstream the first gene.

It has been shown earlier (*15*) that T7 transcripts are largely uncapped, and therefore, their translation must proceed via a cap-independent mechanism, different from the scanning model of translation (*16*). According to the latter model, ribosomes and associated factors bind at or near the 5′-end of the mRNA in a process that is facilitated by the presence of a cap structure, and scan the mRNA until the appropriate initiator codon is reached. The scanning model excludes the possibility of an independent internal ribosome binding site within the 5′-untranslated region (UTR) of the mRNA. However, this rule is contravened in some cases. For example, translation initiation on poliovirus RNA occurs by binding of ribosomes to an internal sequence within the UTR (*17–19*). This highly conserved region (750 bp) contains many AUG codons that probably serve as internal ribosomal entry sites permitting cap-independent initiation of translation (*18*). This alternative mechanism of initiation may explain the translation mechanism of some natural eukaryotic mRNAs that have continuous UTRs containing a few AUG codons that initiate translation of minipeptides in this 5′-upstream region (*20–22*). The influence of the upstream region and small open reading frames on the translation efficiency of poliovirus RNA (*18–23*) and eukaryotic mRNAs has previously been studied in detail. The translation efficiency in both cases is dependent on *cis* effects of defined conserved nucleotide sequences and *trans* effects of protein *trans*-activators (*22*).

UTRs with small open reading frames permit the translation of two or more consecutive coding regions on eukaryotic mRNA (*24*). Vectors with an internal ribosome entry site produce multiple proteins under the control of a single promoter, and multiple transcription units are not expressed. Thus, the potential for promoter suppression is avoided. This is important when the aim is to achieve expression of several heterologous proteins or distinct subunits of a multimeric protein (*24*). As shown in the present work and previously (*8*), T7 RNA polymerase transcribes transfected genes effectively in eukaryotic cells. Effective translation of the mRNA requires appropriate processing of the pre-mRNA transcribed by the T7 polymerase in order to generate mRNA. In several clones transfected with the plasmid pIG.6ek, we detected Ig heavy and light polypeptide chains as discrete bands with molecular size corresponding to the native Ig chains of ϵ- and $\varkappa$-type. The synthesis of functionally active antibodies occurred to comparable extents in lymphoid and nonlymphoid transfected cells. These observations suggest that initiation of translation

in our transfectants occurs by binding of ribosomes to an internal sequence within the 5′-noncoding regions. The upstream UTR sequences in our constructs contain many AUG codons (Fig. 1) and appropriate short reading frames that do not overlap with the reading frames of the immunoglobulin genes located further downstream.

Techniques for the synthesis of recombinant antibodies and chimeric antibodies have been developed in many laboratories (*25,26*). Usually the recombinant antibody genes are expressed in lymphoid cells under the control of natural immunoglobulin gene regulatory elements. In the present work, we have demonstrated the expression of an immunoglobuin gene tandem under the control of the powerful T7 RNA polymerase promoter. This system permits synthesis of recombinant immunoglobulins in nonlymphoid cell lines and should facilitate dissection of factors regulating immunoglobulin gene expression.

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DISCUSSION

S. Deyev

Paul: In the case of the single-chain antibodies, are you working with periplasmic extracts or the medium?

Deyev: We use a periplasmic extract. Inside the cells, the product still contains the signal peptide and is found as an inclusion body. The single-chain antibody without the signal peptide goes to the periplasm. Both types of protein are insoluble. To prepare soluble protein, we used a method of denaturation–renaturation using guanidinium chloride without a reducing agent.

Tramontano: Does the type of host cell influence influence either the yield or the processing of the signal peptide? What was the host cell that you used in your experiment?

Deyev: The host cell was BL21. The signal peptide was obtained from an *Erwinia caratovora* and this signal peptide is processed well in bacteria cells.

Tramontano: Was this a protease-deficient host strain?

Deyev: We have not done a special experiment to test the protease activity in these cells.

McCafferty: BL21 is deficient in protease *Omp*T and *lon*1.

Deyev: It has a *lon* mutation and is a widely used strain.
Stollar: What is the yield of finally refolded soluble antibody?
Deyev: It was about 100 μg/mL. About 20% of the antibody was soluble and effectively bound the original antigen.
Paul: Is the soluble material fully functional?
Deyev: Yes. The specific binding activity of the protein per unit protein was equivalent to the original antibody.
Paul: I am interested in the way Oct2 binds degenerate sequences in the DNA. You are proposing that this binding might facilitate the diffusion of Oct2 along the length of the DNA. Is this a weak type of binding, permitting initial sequence-independent interaction?
Polanovsky: Oct2 has a very high affinity for its natural target sequence as well as the degenerate sequences. The complexes are not dissociated during electrophoresis in retardation experiments.